Analysis of Nonspecific Binding in Dye Affinity Resin Using the Agilent Multiple Affinity Removal System Technology

Application

Bio/Proteomics

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Abstract

The search for biomarkers in proteomics research continues to be a critical bottleneck in the development of new drugs as well as in the study of disease. The traditional methods of analysis to study proteins present in complex mixtures, for example, one- and two-dimensional gel electrophoresis (1DGE and 2DGE), multidimensional liquid chromatography and mass spectroscopic methods, suffer from dynamic range limitations due to the presence of high-abundant proteins. The challenges of analyzing complex protein samples are obvious in the characterization of proteins present in plasma or serum. In such samples, proteins and peptides of interest are present in abundances ranging from half of total protein (for example, serum albumin) to levels less than 10^{-10} of total protein. Although standard liquid chromatography (LC) methods, such as ion exchangers, have been used to fractionate complex protein solutions, a preferred approach is the use of specific interaction media for the selective removal of targeted high-abundant proteins. Dye affinity chromatography resins, exemplified by chlorotriazine dyes, most notably Cibacron Blue (CB), have been used in the removal of albumin from serum samples. An appealing alternative is the use of immunoaffinity chromatographic methods, which can offer extreme specificity and reproducibility. The Agilent Multiple Affinity Removal column selectively removes six of the highest-abundant proteins (albumin, transferrin, haptoglobin, IgG, IgA, and antitrypsin) from human serum with high specificity. This application note demonstrates that a variety of human serum proteins, in addition to serum albumin, bind to the CB resin.

Introduction

The drive to discover novel protein biomarkers in either drug development or disease models is not only vital to further research, but is also a major challenge. The dynamic range of proteins in human serum spans several orders of magnitude with the higher levels representing a smaller number of proteins but at concentrations that can comprise greater than 50% of the total protein mass, such as albumin. Depletion of high-abundant proteins can provide access to previously unreachable proteins. However, many methods either inefficiently remove one or at most two proteins while at the same time suffer from poor specificity. A popular method to remove albumin involves a dye affinity resin, such as CB, or modified versions of this resin. The CB dye has been shown to bind albumin, but has also been used for the binding of NAD, FAD, and ATP binding sites of proteins. This may result in the removal of proteins of interest through a rather broad and nonspecific interaction.

The Agilent Multiple Affinity Removal System is being adopted by proteomic scientists who need to remove multiple high-abundant proteins from human serum or plasma rapidly and simultaneously. The immunoaffinity column simultaneously removes six of the most abundant proteins in human serum using polyclonal antibodies. The Agilent Multiple Affinity Removal column removes greater than 98%-99% of albumin, IgG, IgA,

haptoglobin, transferrin, and antitrypsin from human serum samples as determined by Enzyme-Linked Immunosorbent Assay (ELISA). As previously stated, the immunoaffinity column is based upon polyclonal antibodies, which are purified via a stringent affinity purification method. It can be predicted that the use of highly-purified human antigens for the purification of specific polyclonal antibodies will form a column that only cross-reacts with proteins from closely related species. In addition, this specificity minimizes nonspecific interactions and eliminates the need for resins that are based on cofactor binding sites, which would be more likely to interact with the same proteins from less closely related species as well as other homologous proteins.

This application note shows that CB is rather nonspecific by binding many other proteins in human serum in addition to albumin. The Agilent Multiple Affinity Removal System is quite specific for the targeted proteins. A comparison of the flow-through fractions between CB and the Agilent immuno-affinity column was performed by liquid chromatography tandem mass spectrometry (LC/MS/MS) to show depletion of major proteins. In addition, the bound fraction of CB was analyzed by immunoaffinity to determine the additional proteins bound nonspecifically to CB versus single immunoaffinity columns in the Agilent Multiple Affinity Removal System.

Experimental

The Multiple Affinity Removal System for removing albumin, transferrin, IgG, IgA, haptoglobin, and antitrypsin from human serum is a product from Agilent Technologies (Wilmington, DE). A 4.6 mm × 100-mm Multiple Affinity Removal column was used with a mobile phase reagent kit (Agilent part number 5185-5985, and 5185-5986). Sample loading, washing and column regeneration is done using Buffer A and for bound protein elution, Buffer B is used according to manufacturer protocols. Injections of diluted serum were performed according to manufacturer protocols for a 4.6 mm × 100-mm column. Fractions were automatically collected by time into 1.5-mL plastic tubes (Sarstedt, Numbrecht, Germany) using an Agilent 1100 HPLC equipped with a thermostatted analytical scale fraction collector. Depleted low-abundant proteins as well as high-abundant bound proteins were collected and stored at –20 °C until analysis.

Cibacron Blue

Serum was diluted 4-fold with 50-mM K_2HPO_4 pH 7 (load buffer). The sample was loaded onto a Hi Trap Blue HP Column (Amersham Biosciences, Piscataway, NJ) and washed with five column volumes using the load buffer. The proteins, which did not bind to CB, were collected and labeled as flow-through. Proteins that were bound to CB were eluted using 50-mM K_2HPO_4 /1.5 M KCl, pH 7. Both the flow-through and the bound fractions from several runs were collected, pooled and concentrated.

Analysis of CB Bound Fraction

The concentrated bound fraction from CB was diluted to the appropriate concentration with Buffer A for loading onto a specially prepared anti-Human Serum Albumin (anti-HSA) column. The anti-HSA column is packed with only the anti-HSA antibody used in the Agilent Multiple Affinity Removal column. Several injections of the CB bound fractions were loaded onto the anti-HSA column and flow-through fractions were collected and analyzed by 1D gel and LC/MS/MS.

LC/MS Analysis

The CB flow-through fraction was digested with trypsin and analyzed by 2D LC/MS/MS on an Agilent 1100 MSD Trap SL. Data analysis for all LC/MS experiments was performed with Agilent Spectrum Mill Software. Similar analyses were performed on crude serum and the flow-through fractions from the Agilent Multiple Affinity Removal column. Briefly, a SCX column (Polysulfoethyl A, 1 × 50 mm, 5 µm, 300 Å from PolyLC) was used for the first dimension. A Zorbax 300SB-C18 5 µm, 0.3 × 150-mm column was used for the reversed phase separation. The loading flow rate was 0.1 mL/min using 3% ACN/0.1% formic acid. For reverse phase analysis, Solvent A was 0.1% formic acid and solvent B was 100% ACN/0.1% formic acid. The flow rate for reverse phase was 6 µL/min. The gradient for reverse phase and the salt slices are as follows:

Gradient: 10% B for 5 min; 90 min 60% B; 92 min 90% B; 97 min 90% B; 100 min 10% B

Salt slices: 10 mM, 25 mM, 50 mM, 75 mM, 100 mM, 200 mM, 500 mM, 1 M, and 2.5 M KCl

Buffer Exchange with Spin Concentrators

To resolve bound fractions on 1D gels, fractions were placed in 4-mL spin concentrators with a

5 kDa molecular weight cutoff (MWCO); (Agilent part number 5185-5991) and filled to maximum volume with Buffer A. The sample was centrifuged at 7,500 × g for 20–25 minutes at 8 °C. This process was repeated three times for complete buffer exchange. Buffer-exchanged samples were placed in a clean Eppendorf tube for storage. Protein concentrations for crude serum, flow-through fractions and bound fractions (buffer exchanged) were determined using a Pierce BCA protein assay kit.

Protein Analysis by SDS-PAGE

The protein patterns of serum samples before and after depletion were visualized on sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) gels. The bound fractions were buffer-exchanged three times into Buffer A to minimize sample manipulation of the low-abundance flow-through fraction. Equal amounts of protein mass from each fraction and from crude serum were resolved on Novex 4%–20% Tris-Glycine gels (Invitrogen, Carlsbad, CA) under nonreducing conditions. Proteins were visualized by Coomassie Blue staining.

Results and Discussion

In order to determine the specificity of CB, a separate anti-HSA immunoaffinity column was produced using the same technology as the Agilent Multiple Affinity Removal column. A sample of serum is then loaded onto the CB column, the column is washed and the bound fraction on CB is eluted and collected. The bound fraction is then diluted with a phosphate buffer and loaded onto the anti-HSA immunoaffinity column to deplete albumin while proteins that bound nonspecifically to CB will pass through and be collected as the flow-through fraction (Figure 1).

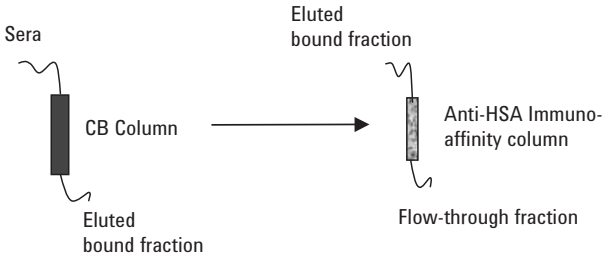

Figure 1. Experimental design to determine proteins nonspecifically bound to CB during albumin depletion.

The bound fraction of CB was buffer exchanged and proteins were visualized by running on 1DGE (Figure 2). In addition, flow-through samples from CB bound fractions that were depleted of

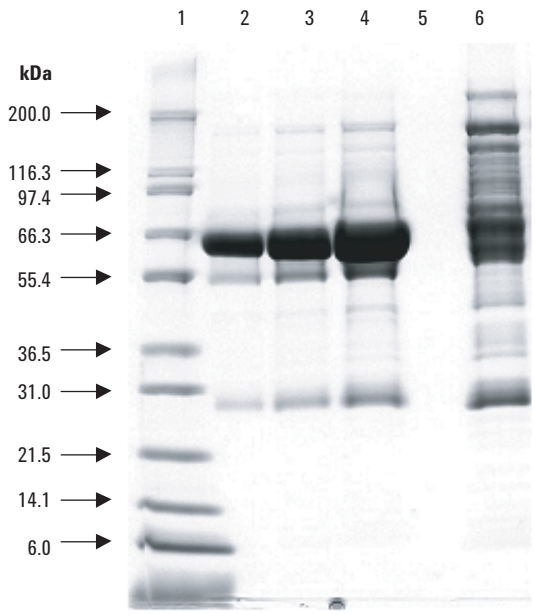

Figure 2. Analysis of CB fractions by 1DGE. Lane 1 - Molecular weight standards; Lane 2 - Bound fraction, 2 µg; Lane 3 - Bound fraction 4 µg; Lane 4 - Bound fraction, 6 µg; Lane 5 - Blank; Lane 6 - CB bound flow-through fraction from anti-HSA immunoaffinity column.

almbumin by the anti-HSA column were visualized by 1DGE. The results indicate that there are many proteins that bind nonspecifically to CB that are not bound by the anti-HSA column.

Albumin was removed from the bound fraction of the CB column using an anti-HSA column and the flow-through fraction was analyzed by 1DGE and LC/MS/MS. The list in Table 1 shows an extensive number of proteins that bind nonspecifically to the CB column.

The specificity of the Agilent Multiple Affinity Removal column was examined by using single immunoaffinity columns for each of the targeted proteins, such as an anti-IgG column. Serum was loaded onto the column and the bound fractions were collected, buffer exchanged and resolved on 1D gels. Bands from the bound fractions of six individual immunoaffinity columns were excised and digested and analyzed by LC/MS/MS. A list of proteins that bind to individual immunoaffinity columns for each of the six targeted proteins in the Agilent Multiple Affinity Removal system is shown in Table 2. The proteins listed in bold were those that may bind nonspecifically to the Multiple Affinity Removal column. However none of the nonspecific proteins were bound quantitatively to the columns as estimated by LC/MS.

Table 1. List of proteins that bind to CB in addition to albumin as determined by LC/MS/MS. Proteins in bold are removed quantitatively by the CB column.

Number	Protein name	Number	Protein name
1	Alpha2-macroglobulin	29	Peptidoglycan recognition protein L
2	**Complement C3**	30	Insulin-like growth factor binding protein
3	**Complement C4**	31	Ig lambda light chain VLJ region
4	Ceruloplasmin	32	Haptoglobin-related protein
5	Alpha1-antitrypsin	33	α–1 microglycoprotein
6	Serum albumin precursor	34	Afamin precursor
7	α-1-antitrypsin	35	Ig heavy chain variable region
8	Apolipoprotein 1A	36	Trypsinogen hL
9	Antithrombin III	37	Ig alpha heavy chain variable region
10	**ITIH2**	38	Ig kappa light chain VLJ region
11	**ITIH1**	39	Cytokeratin 9
12	C1 inhibitor	40	Trypsinogen 16
13	**ITIH4**	41	Complement C6
14	**Hemopexin**	42	ATP-binding
15	Complement factor B	43	Angiotensin
16	Kininogen LMW	44	Complement C5
17	Ig mu chain	45	KIAA1461 protein
18	Gelsolin	46	Ig lambda light chain variable region
19	Ig kappa light chain	47	ATP synthase F0 subunit 6
20	Heparin cofactor II	48	Ig heavy chain-VIII region HIL
21	Serum amyloid P component	49	Hypothetical protein XP_289343
22	Trypsin precursor (pig)	50	S-protein precursor
23	Complement component C7	51	Embryonicleucine zipper kinase
24	Complement 9	52	Cul-3
25	Ig heavy chain	53	Unnamed protein product
26	Alpha2 antiplasmin	54	Coagulation factor X precursor (Stuart factor)
27	Clusterin	55	Serum aryldiakylphophatase1
28	Ig alpha1 chain C region	56	Complement C1s
		57	TFNR

Table 2. List of proteins that bind to individual immunoaffinity resins from the Multiple Affinity Removal Column. Proteins in bold represent nontargeted proteins that were identified but were not bound quantitatively to the column.

Number	Protein name	Number	Protein name
1	Serum albumin	13	Ig alpha-1 chain C region
2	Serotransferrin	14	Ig kappa chain V region
3	Ig heavy chain 4	15	**Apolipoprotein A-1**
4	α-1-antitrypsin	16	Ig heavy chain V region
5	**Alpha-2-macroglobulin**	17	Ig mu heavy chain variable region
6	Ig M heavy chain	18	**Complement C4**
7	Haptoglobin	19	Ig light chain variable region
8	Ig kappa light chain VLJ region	20	Ig kappa chain V-IV region (Len)
9	Ig lambda light chain VLJ	21	Ig alpha heavy chain variable region
10	**Hemoglobin**	22	Ig light chain variable region
11	**Complement C3**	23	Ig heavy chain VHDJ region
12	**Transthyretin**		

The results show that the Agilent Multiple Affinity Removal System is specific for the targeted high-abundant proteins, including albumin, transferrin, IgG, IgA, haptoglobin, and antitrypsin. When the CB bound fraction was analyzed, the data indicated that many additional proteins were bound to the resin and some were quantitatively bound as shown in Table 1.

A comparison of proteins identified in serum, as well as serum depleted with CB or serum depleted by the immunoaffinity column, is shown in Table 3. The proteins with names labeled in bold indicate those that are lost in the CB flow-through fraction due to binding to the column packing material with albumin. The proteins with names labeled in italics indicate proteins that should be removed or reduced with the Multiple Affinity Removal column. The different shades in the columns to the left are an indication of the relative abundance of the protein present in the sample as determined with Agilent Spectrum Mill software (darker means more abundant).

Further evidence for nonspecific binding in CB is found by analyzing the protein hemopexin which was identified in serum, as well as in immunodepleted serum. This protein is typically masked by albumin in gels yet is identified by MS in immunoaffinity depleted serum, as well as in crude serum, whereas CB nonspecifically removes this protein. The extracted ion chromatograms (EICs) for a peptide of hemopexin MW 3282, found in the immunoaffinity flow-through fraction and sera, but not found in the CB flow-through fraction, are shown in Figure 3. The 2+, 3+, and 4+ ions were observed in both sera and immunoaffinity flow-through fractions. The hemopexin peptide was observed in the CB bound fraction (data not shown). Similar results were observed for other hemopexin peptides.

Table 3. Comparison of proteins identified with relative quantitation of serum, Agilent Multiple Affinity Removal-depleted serum and CB-depleted serum using Agilent Spectrum Mill Software. Proteins in bold are removed by CB, proteins in italics are targeted by the Agilent Multiple Affinity Removal System and the shaded boxes represent relative quantities of protein in each sample. It is important to note not only the relative abundance but to note the number of spectra used in the identification of the proteins, for example, IgG1 shows 34 spectra identified in serum, 78 identified in CB flow-through fraction and only 1 spectrum identified in the immunoaffinity flow-through fraction.

CB # spectra intensity	Affinity # spectra intensity	Sera # spectra intensity	#	Protein Name
59 5.13e+008	0 0.00e+000	1146 3.06e+010	1	*Serum albumin*
153 5.76e+009	0 0.00e+000	42 3.32e+008	2	*Serotransferrin*
58 8.87e+008	98 1.94e+009	37 3.30e+008	3	Alpha-2-macroglobulin
7 4.22e+007	100 1.06e+009	26 1.07e+008	4	**Complement C3**
109 4.36e+009	14 1.67e+008	34 7.70e+008	5	*Alpha-1-antitrypsin*
28 6.52e+008	26 3.57e+008	9 4.76e+007	6	Vitamin D-binding protein
30 1.88e+009	0 0.00e+000	16 2.35e+008	7	*Haptoglobin*
0 0.00e+000	87 5.55e+008	19 2.94e+008	8	**Complement C4**
78 4.43e+009	1 2.59e+006	34 1.49e+009	9	*IgG1*
21 2.09e+008	68 1.30e+009	28 2.53e+008	10	Apolipoprotein A-I
69 1.01e+009	5 4.02e+007	12 1.76e+008	11	*Ig alpha*
0 0.00e+000	24 1.8e+008	7 2.45e+007	12	**Hemopexin**
25 1.72e+009	2 2.57e+007	12 3.65e+008	13	*Immunoglobulin kappa*
0 0.00e+000	17 8.19e+007	6 8.79e+006	14	**Fibronectin**
50 7.36e+008	57 5.27e+008	18 1.15e+008	15	Transthyretin
0 0.00e+000	11 1.27e+008	3 2.03e+006	16	**ITIH1**
0 0.00e+000	28 9.32e+007	5 1.69e+007	17	**ITIH2**
0 0.00e+000	12 1.24e+008	0 0.00e+000	18	**ITIH4**
9 4.45e+007	3 4.16e+007	10 5.04e+007	19	Ig mu
19 3.52e+008	14 7.47e+007	7 4.85e+007	20	α–2–HS glycoprotein
0 0.00e+000	32 2.50e+008	8 1.23e+008	21	**B - factor, properdin**
9 1.21e+008	12 6.93e+007	3 6.76e+006	22	Prothrombin
6 4.70e+007	74 1.16e+009	10 1.26e+008	23	**Apolipoprotein A-II**
1 3.03e+006	11 9.66e+007	2 8.40e+006	24	**Cerulopdlasmin**
19 1.66e+009	5 1.19e+008	25 2.09e+009	25	*Ig lambda*
8 4.63e+007	0 0.00e+000	0 0.00e+000	26	Zn-α–2–glycoprotein
14 8.80e+007	8 1.19e+008	1 5.22e+006	27	Orosomucoid 1
0 0.00e+000	22 1.36e+008	1 8.35e+006	28	**Alpha-1-antichymotrypsin**
0 0.00e+000	8 5.92e+007	1 1.52e+006	29	**Plasma protease C1 inhibitor**
0 0.00e+000	8 2.60e+007	4 7.25e+006	30	**Complement factor H**
8 9.03e+007	8 1.03e+008	4 4.10e+007	31	Alpha 1B-glycoprotein
0 0.00e+000	4 1.55e+007	2 1.31e+006	32	**Kininogen, LMW**
0 0.00e+000	4 2.58e+007	6 3.78e+007	33	**Apolipoprotein B-100**
3 2.18e+007	0 0.00e+000	0 0.00e+000	34	Lumican
0 0.00e+000	4 5.77e+007	3 9.48e+006	35	Clusterin
0 0.00e+000	3 5.74e+007	2 4.35e+007	36	Apolipoprotein H
4 5.23e+007	1 1.72e+007	0 0.00e+000	37	Leucine-rich α–2–glycoprotein
5 6.00e+007	1 2.85e+006	4 8.83e+006	38	Trypsin
0 0.00e+000	8 3.35e+007	4 1.30e+007	39	**Complement C5**
2 4.45e+007	0 0.00e+000	1 2.61e+007	40	Ig Kappa
2 5.90e+006	1 1.30e+007	0 0.00e+000	41	Angiotensinogen
2 3.04e+006	7 3.46e+007	0 0.00e+000	42	Antithrombin-III
0 0.00e+000	3 6.39e+006	2 4.78e+006	43	Carboxypeptidase
0 0.00e+000	1 1.03e+006	4 7.28e+006	44	Platelet factor 4
0 0.00e+000	1 5.46e+006	1 1.83e+006	45	Plasmin
4 1.12e+007	0 0.00e+000	0 0.00e+000	46	Beta globin chain variant
1 1.75e+007	0 0.00e+000	1 1.06e+007	47	Ig kappa chain V-I region
1 5.31e+007	0 0.00e+000	1 2.61e+007	48	Immunoglobulin kappa

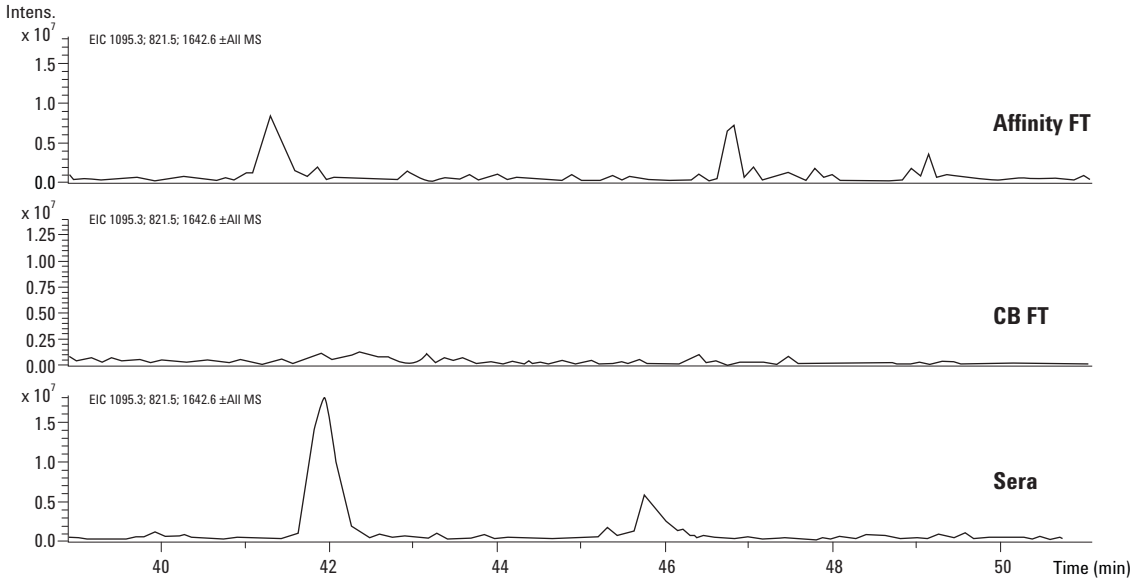

Figure 3. EICs for a hemopexin peptide with MW 3282. The top panel shows the peptide is present in the Agilent immunoaffinity flow-through fraction, similar to the bottom panel for serum. The peptide is lost in the middle panel representing the CB flow-through fraction, indicating that it is retained on CB.

Conclusions

The Agilent Multiple Affinity Removal System shows remarkable specificity for six high-abundant proteins in human serum. The immunoaffinity column has proven quite specific to its targeted proteins. The CB column has extensive nonspecific binding as determined by analysis of the bound fraction with an anti-HSA immunoaffinity column. The results of this study show that the specificity and selectively of the Multiple Affinity Removal System is uniquely optimized for human proteins and is consistent with previous studies showing the highest possible selectivity for the targeted proteins and low interaction with nontargeted proteins. This technology will enable proteomic scientists in the search for novel biomarkers to discover more low-abundant proteins than permitted by previous nonspecific and inefficient technologies.

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